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2. Patent application number *(The Patent Office will fill in this part)* — **9917180.3**

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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

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4. Title of the invention — CELL SURFACE RECEPTOR

5. Name of your agent *(if you have one)*

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6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
   a) any applicant named in part 3 is not an inventor, or
   b) there is an inventor who is not named as an applicant, or
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YES

CELL SURFACE RECEPTOR

The invention herein described relates to cells and/or tissues and/or organs which do not naturally express the cell surface receptor CD40 ligand, CD154, for use, particularly but not exclusively, in therapeutic and cosmetic tissue engineering and/or organ transplantation; compositions comprising said cells and/or tissues; organs comprising said cells/tissues; and methods of therapy and/or cosmetic surgery using said cells and/or tissues and/or organs.

Tissue engineering is an emerging science which has implications with respect to many areas of clinical and cosmetic surgery. More particularly, tissue engineering relates to the replacement and/or restoration and/or repair of damaged and/or diseased tissues to return the tissue and/or organ to a functional state. For example, and not by way of limitation, tissue engineering is useful in the provision of skin grafts to repair wounds occurring as a consequence of: contusions, or burns, or failure of tissue to heal due to venus or diabetic ulcers. Further, tissue engineering is also practised during: replacement of joints through degenerative diseases such as arthritis; replacement of coronary arteries due to damage as a consequence of various environmental causes (e.g. smoking, diet) and/or congenital heart disease including replacement of arterial/heart valve; repair of gastric ulcers; replacement bone tissue resulting from diseases such as osteoporosis; replacement muscle and nerves as a consequence of neuro muscular disease or damage through injury.

In addition, organ transplantation has for many years been an established surgical technique to replace damaged and/or diseased organs. The replacement of heart, lung, kidney, liver, bone marrow, and double organ

The body has developed many defences against invasion of foreign organisms. These humoral and cellular defence mechanisms are also directed against foreign antigens expressed by various tissues/organs used in tissue engineering and/or organ transplantation.

A general term to cover a number of distinct cell types intimately involved in both a humoral and cellular defence mechanism are the white blood cells. Each white blood cell type has a separate role to play in a hosts immune system. Monocytes are large white blood cells that differentiate into macrophages. The macrophages are found throughout the body in various types. For example specialised macrophages include alveolar macrophages in the lungs, mesangial phagocytes in the kidneys, microglial cells in the brain, and Kuppfer cells in the liver. Macrophages have many roles and these include, by example and not by way of limitation, ingestion of infectious agents, antigen presentation to T-lymphocytes and the secretion of agents involved in regulating the immune system (i.e. interlukin-1, complement proteins).

A pivotal cell-cell interaction between the many cell types which comprise the immune system is between T- lymphocytes (T- cells) and B- lymphocytes (B- cells). T – cells recognise polypeptide antigens presented as peptides via self molecules referred to as the major histocompatibility complex (MHC) on antigen presenting cells such as macrophages. T-cells are divided into cytotoxic T- cells (CTL's) and T- helper cells. The latter class of T-cell are able to stimulate B- cell proliferation and mediate immunoglogulin isotype switching to produce antibody isotypes (IgG, IgA, IgD, IgM, IgE) to specific peptide antigens.

Given the importance of the interaction of CD40 receptor with CD154 we have undertaken a study of the interaction between these molecules by expressing CD154 in cells which do not naturally express CD154, namely mouse fibroblasts expressing a mismatched MHC class, and used these cells as an immunogen. We anticipated that this would act as a potent stimulator of the immune system.

It is known that blocking the interaction of CD40 with CD154 can supress the immune response. For example, the use of anti- CD154 antibodies is known to abrogate the interaction between CD40 receptor and CD154 and result in attenuation of the immune system in response to allografts (WO9856417 & WO9858669); suppression of autoimmune disease (WO9900143) and blood clotting disorders (WO9858672). We predicted that the recombinant expression of CD154 in MHC mismatched cells would promote an immune response to said cells. To our surprise, these cells did not promote an immune response but resulted in immune suppression toward the injected transfected fibroblasts.

It is apparent that this observation has important implications with respect to allotypic recognition of implanted cells/tissues/organs. The expression of CD154 in cell types which do not naturally express this ligand resulted in failure of the immune system to recognise the implanted cells as foreign. This has important implications with respect to tissue/organ transplantation and tissue/organ rejection by the host receiving the transplanted tissue/organ.

It is an object of the invention to provide cells/tissues and/or organs which when implanted into a host exhibits immunoprivilige ie they appear not to be recognised by the host immune system.

In yet still a further preferred embodiment of the invention said cells/tissues are selected from the following cell types; fibroblast; keratinocyte; osteoblast; chondrocyte; neurones, myocytes; hepatocytes; splenocytes, pancreatic β cells.

According to a second aspect of the invention there is provided a vector for use in the transfection of a selected cell/tissue/organ type for use in tissue engineering and/or organ transplantation characterised in that it contains a DNA molecule encoding at least the effective part of CD154 ligand, or a homologue thereof.

In a preferred embodiment of the invention said vector is adapted for the recombinant expression of CD154 ligand.

Conventionally, nucleic acid molecules used to transfect cells are referred to as vectors. Vectors used in genetic engineering are typically circular molecules, (although some may be linearised prior to transfection to facilitate the introduction of DNA into a host cell). Vectors of this type are referred to as plasmids, phages, or phagemids. In many examples these vectors have been genetically engineered to adapt them for expression in eukaryotic cells. For example, and not by way of limitation the provision of cell/tissue specific promoter elements which facilitate expression in a specific cell/tissue type; the provision of viral promoters which provide high levels of constitutive expression.

In addition to the above identified vectors, viral based vectors are used in transfection and in particular, gene therapy, to deliver genes to tissues *in vivo*. These vectors typically retain the capability to infect a host cell but are genetically modified to render the virus biologically disabled, this latter

In a preferred embodiment of the invention said cell/tissue/organ is selected from the following tissue types: neuronal, muscle (e.g. smooth, striated, cardiac), bone, cartilage, liver, kidney, respiratory epithelium, endothelium, haematopoietic cells, spleen, pancreas, skin, stomach, intestine, oesophagus;blood vessels.

According to a third aspect of the invention there is provided a method to transfect a selected cell/tissue comprising:

i. incubating cells/tissue under conditions conducive to the introduction and maintenance of a vector according to the invention;

ii. exposing said cells to an agent at a concentration sufficient such that at least those cells/tissues including said nucleic acid molecule are resistant to said agent; and optionally,

iii. culturing said cells/tissues containing said nucleic acid molecule and, optionally, further still,

iv. storing said cell culture prior to use.

In a preferred method of the invention said cell/tissue is a mammalian cell/tissue. Ideally said mammalian cell/tissue is of human origin.

In a further preferred method of the invention, said transfection is transient. In the event that a transient transfection is required; steps (ii) and (iii) are not necessary.

Conditions which would benefit from therapeutic tissue engineering include by example, and not by way of limitation, arthritis and the replacement of joints; skin grafting for burn victims or injuries resulting in sever contusions; replacement of coronary arteries; replacement of diseased or damaged nerves and/or muscles; replacement of pancreatic β cells.

According to a fifth aspect of the invention there is provided at least one organ wherein said organ for use in organ transplantation comprises at least one cell/ tissue according to any previous aspect or embodiment of the invention.

In a preferred embodiment of the invention said organ comprises at least one cell/tissue transfected with the vector according to any previous aspect or embodiment of the invention.

According to a sixth aspect of the invention there is provided a cell/tissue composition for use in cosmetic tissue engineering comprising at least one cell/tissue according to any previous aspect or embodiment of the invention.

In a preferred embodiment of the invention said cell/tissue comprises at least one cell/tissue transfected with the vector according to any previous aspect or embodiment of the invention.

According to an seventh aspect of the invention there is provided a method of treatment comprising;

i) providing at least one cell/tissue which does not normally express CD154, or effective part thereof, and adapted so that same expresses at least the effective part of CD154;
ii) administering said cells/tissues to a patient to be treated; and optionally;

Figure 2 represents groups of five BALB/c mice immunised with L cells or CD154 L cells and boosted with either L cells or CD154 L cells;

Figure 3 represents groups of five BALB/c mice immunised with CD154 L cells or PBS, immunised again with normal L cells 3 and 6 weeks later and bled 8 days after the last immunisation;and

Figure 4 represents measurements of serum nitrate levels of mice after immunisation with L cells or CD154 L cells.

MATERIALS AND METHODS

Cells and antibodies

L929 cells (L cells) and CD154 transfected L929 cells (CD154 L cells) were kindly provided by DNAX Research Institute, California. CD154 transfected L929 cells were prepared as described elsewhere [15]. Anti-CD40 antibody 1C10 (9) was purified on a protein G column from hybridoma supernatant produced in a bioreactor by Sheffield hybridomas, Sheffield. The MR1 anti-CD154 mAb was purchased from Pharmingen.

Mice and Immunisations

BALB/c female mice of 8-12 weeks of age were obtained from the University of Sheffield Field Laboratories. L929 cells were removed from tissue culture flasks using EDTA (0.5mM), washed in PBS and 5 x 10^4 cells injected intraperitoneally into MHC mismatched BALB/c mice. Mice were bled 10 days post-primary immunisation and seven days after each subsequent immunisation.

The antibody responses of BALB/c mice against normal or CD154 expressing L929 cells were not detectable by this flow cytometric assay following a single immunisation. However two i.p injections of L cells gave rise to antibody responses against L cells of around 1/1000 (Figure 1). In contrast mice immunised with CD154 L cells produced no detectable response against L cells even after two immunisations (Figure 1). We considered it possible that these apparent differences in immunogenicity were caused by antigenic variation between the two cell lines as responses were assayed against normal L cells; however similar results were seen when CD154 L cells were used as the antigen in the assay, Figure. Thus the results were due to a lack of response to the CD154 L cells in immunised mice.

CD154 L cells fail to prime for an alloantibody response against normal L cells, but do not induce tolerance.

Mice immunised first with CD154 L cells, and then with normal L cells failed to produce a normal secondary antibody response against the L cells, thus CD154 expression inhibited priming of the antibody response against L cells ($p=0.013$; Fig 2). It was possible that the CD154 L cells were inducing tolerance, leading to a lack of response on secondary exposure to normal L cells. To determine whether this was the case, two groups of mice were immunised with two doses of L cells, but one of the groups had previously been immunised with CD154 L cells. Had the CD154 expressing cells induced tolerance then the latter group should have produced a lower immune response. In fact this was not the case, and responses between the two groups were the same ($p=0.23$; Fig 3). Thus it would appear that CD154 expression does not result in tolerance, but rather a lack of recognition of the antigen on initial exposure.

Induction of Nitric oxide by CD154 expressing L929 cells

DISCUSSION

Interactions between CD154 and CD40 play a very important role in immune responses. In general, stimulation of B cells through CD40 induces strong B cell activation, proliferation and isotype switching especially in co-operation with signalling by other factors such as antigen (or anti-IgM), and cytokines such as IL4. Signalling through CD40 also appears to be important in initiating and maintaining germinal centres [1]. Activation of B cells through anti-CD40 antibodies in vivo can also give rise to enhanced isotype switching, greatly increased antibody responses [6,11] and B cell proliferation [Dullforce, Greenwood and Heath, in preparation]. As we had demonstrated strong adjuvant-like effects of CD40 ligation on antibody responses *in vivo*, we considered that cell-surface expression of the CD40 ligand, CD154, may be a potent means of enhancing anti-cellular immune responses. An analogous approach had been used successfully utilising CD80 and CD86 transfection to enhance the CTL response against tumour cells in vivo [12]. We therefore examined the effect of transfection with CD154 on the alloantibody response to murine L929 cells.

BALB/c mice (H-2^d) were immunised with the MHC mismatched cell line L929, untransfected or stably transfected and expressing murine CD154 on the membrane. Contrary to our expectations we found that expression of CD154, rather than enhancing the alloantibody response to the L929 cells, actually suppressed the response. This suppression only became evident after two immunisations because of the poor primary response to normal L cells. However, the suppressive effect was mediated at the primary immunisation as the response to a second injection with normal L cells was suppressed by primary immunisation with CD154 L cells. The lack of responsiveness to CD154 L cells was not caused by antigenic differences between the two cell lines, as similar results were obtained when CD154 L cells were used as antigen to detect the antibody. It appeared possible that the CD154 expression was rendering the L cells

might be expected to be present in the periphery. Because of the function of CD154, a CD154 specific B cell coming across it for the first time would receive a potent "danger" signal in the form of CD40 ligation. Thus it might be expected that strong autoantibody responses against CD154 would be produced. This is clearly not the case, and we propose that simultaneous and long-lived stimulation through surface immunoglobulin and CD40 and/or extensive cross-linking of the two receptors, may anergise the B cell preventing the production of large amounts of autoantibody against CD154 and possibly TCR idiotopes.

We consider that transfection of donor cells for transplantation with CD154 will have some role to play in enhancing the acceptance of allografts by the recipient.

REFERENCES

1) Kawabe, T., Naka ,T., Yoshida, K., Tanaka, T., Fujiwara, H., Suematsu, S., Yoshida, N., Kishimoto, T and Kikutani, H. . Immunity 1, 167 1994

2) Xu J.C., Foy T.M., Laman J.D., Dunn J.J., Waldshmidt T.J., Elsemore J., Noelle R.J. and Flavell R.A. Immunity 1 423, 1994

3)Yang., Y and Wilson, J.M. Science 273, 1862, 1996

4) Van Essen, D., Kikutani, H and Gray, D. Nature 378, 620, 1995

5)Armitage, R.J., Fanslow, W.C., Strockbine, L., Sato, T.A., Clifford, K.N., Macduff, B.M., Anderson, D.M., Gimpel, S.D., Davis-Smith, T., Maliszewski, C.R., Clark, E.A., Smith, C.A., Grabstein, K.H., Cosman, D and Spriggs, M.K. Nature 357, 80, 1992

6) Dullforce, P., Sutton, D and Heath, A.W. Nature Medicine 4 , 88, 1998

7) Tian,L.C., Noelle, R.J. and Lawrence, D.A. Eur. J.Immunol. 25:306, 1995